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Evidence for Non-steroidal Gonadal Regulator(s) of Gonadotropin Release in the Goldfish, *Carassius auratus*

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ABSTRACT—In mammals, it is well documented that in addition to steroidal hormones, gonads also produce a variety of non-steroidal proteins and peptides that can feed back to regulate pituitary gonadotropin secretion. The best studied among these non-steroidal substances are inhibin and activin. The present study demonstrates that in sharp contrast to mammalian gonadal extracts which usually have inhibitory effects on mammalian gonadotropin secretion due to inhibin, the aqueous goldfish gonadal extracts stimulate pituitary gonadotropin-II (GTH-II) release in goldfish. Interestingly, the actions of the goldfish gonadal extracts are similar to our previously reported effects of porcine follicular fluid on goldfish GTH-II release. The stimulatory activities of goldfish ovarian extracts and the pituitary resopnsiveness to this stimulation fluctuate dramatically with the ovarian development. The results presented in this paper support our previous findings that porcine inhibin and activin stimulate goldfish gonadotropin release and the immunoreactive inhibin and activin subunits are present in the goldfish gonads.

INTRODUCTION

McCullagh first described 60 years ago that animal gonads produced non-steroidal substances that influenced gonadotropin (GTH) secretion. The term "inhibin" was proposed to designate a putative factor in the aqueous extracts of bull testes that could prevent the appearance of castration cells in the anterior pituitary gland [20]. The putative gonadal "inhibin" was later demonstrated to be a selective non-steroidal inhibitor of follicle-stimulating hormone (FSH) secretion. During the past decades, inhibin activity of suppressing basal FSH release has been demonstrated in various gonadal preparations such as follicular fluids [5–7, 13, 17, 21, 35, 40], ovarian extracts [2, 3, 36], testicular extracts [12, 23, 33, 36], rete testis fluids [1] and seminal plasma [4, 32] from a variety of mammalian species including pigs, cows, sheep, rodents, horses and humans.

With the development of a specific biossay to monitor the purification process for inhibin, four laboratories simultaneously purified inhibin molecules from porcine [15, 22, 28] and bovine follicular fluid [29] in 1985. During the separation of inhibin from porcine follicular fluid, another novel protein was isolated that stimulated FSH release from cultured rat pituitary cells and was named FSH releasing protein (FRP) [38] or activin [16]. Biochemical characterization revealed that activin is structurally related to inhibin [16, 38]. The stimulatory actions of activin in the gonadal preparations are believed to be overridden and masked by the potent inhibitory actions of inhibin on FSH release, which results in an overall inhibitory effect of the aqueous extracts from gonads. In addition to inhibin and activin, a variety of other protein or peptide factors of gonadal origin which had either stimulatory or inhibitory effects on pituitary GTH release have been identified in mammals [14, 18, 27, 31, 32, 34, 42]; however, studies on these factors are fragmentary compared to those on inhibin and activin.

The purpose of this study was to provide preliminary evidence for the presence of non-steroidal substances in the goldfish gonads that may regulate pituitary GTH secretion. The present work serves as an initial point for future studies on the identities of such molecules. A protocol similar to that used in mammals was adopted in the present work; however, a pituitary fragment perifusion system was used as the bioassay instead of static culture of pituitary cells. In common carp, a closely related species to goldfish, two chemically distinct GTHs have been identified and designated GTH-I and GTH-II; however unlike mammalian FSH and luteinizing hormone (LH), which have distinct biological functions, carp GTH-I and GTH-II possess the same biological activites in goldfish, in terms of stimulation of gonadal steroidogenesis and induction of oocyte final maturation [39]. Since the radioimmunoassay for GTH-I was not available, the present study was based on the measurement of GTH-II using carp GTH-II as the standard in the assay.

MATERIALS AND METHODS

Experimental animals

Common or comet variety goldfish (*Carassius auratus*) of mixed sex (approximately 25 g) were purchased from Grassyfork Fisheries Co., Martinsville, IN, USA or Ozark Fisheries, Stoutland, MO, USA. Fish were acclimated to 17±1°C and a simulated natural photoperiod of Edmonton, and fed a commercial trout food, for at least one week before use. Animals were anaesthetized with 0.05% tricaine methanesulphonate before handling.

Tissue extracts

Mature goldfish ovaries were homogenized with a Polytron homogenizer and extracted in phosphate-buffered saline (PBS, 50 mM+0.8% NaCl, pH 7.4) (1 : 1.5, weight:volume) containing 0.01 M

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phenylmethylsulfanyl fluoride (PMSF, a proteinase inhibitor) for 3 hr at 4°C. The homogenates were centrifuged for 1 hour at 20,000×g. The supernatants were treated with Dextran-coated charcoal (30 mg/ml charcoal, 3 mg/ml Dextran) for 30 min to remove endogenous steroids. After centrifugation, the supernatants were dialysed (with 12 kD cut-off) against double distilled water for 24 hr, lyophilized, and stored at −20°C for subsequent use. Testosterone and estradiol levels in the crude goldfish ovarian extract (CGOE) were 0.12±0.05 and 0.20±0.11 ng/10 mg CGOE, respectively.

Extracts from the ovaries of sexually regressed and recrudescing fish, testes and muscles were prepared following the same procedures described above. The ovarian fluid was collected by centrifuging the ovulated eggs. CGOEs prepared from regressed, recrudescing and mature ovaries are designated CGOE-reg, CGOE-rec and CGOE-mat, respectively. The ovary was judged at dissection as regressed if no vitellogenic oocytes were visually evident, as recrudescing if vitellogenic oocytes could be seen, and as mature (=prespawning) if the ovary appeared full of large yolky oocytes.

Perifusion of pituitary fragments

For bioassay, an *in vitro* pituitary perifusion system was used as described by Marchant *et al.* [19]. Briefly, the whole pituitaries were collected from goldfish of mixed sexes and cut into small fragments (<0.05 mm^3) with a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Guildford, Surrey, England). The fragments were then washed three times with medium M199 and loaded between two layers of Cytodex beads (Pharmacia, Uppsala, Sweden) in mini-columns (3 pituitaries/column). The fragments were perifused with medium M199 with Hank's salts, 0.35 g/litre $NaHCO_3$, 25 mM Hepes, antibiotics and 0.1% BSA (M199-H-BSA, pH 7.2) at 17°C. The effects of long-term and short-term exposure to CGOE of the perifused pituitary fragments on GTH-II secretion were examined. For the long-term approach, the pituitary fragments were exposed to CGOE-mat-containing medium (1 mg/ml) for 24 hr at the perifusion rate of 5 ml/hr. The perifusion rate was increased to 15 ml/hr 2 hr before collecting the media in fractions, and the treatment with CGOE continued. After the fraction collection started, a series of 2 min 20 nM salmon gonadotropin-releasing hormone (sGnRH) pulses were applied at 60 min intervals. The treatment with CGOE-mat was terminated after the second pulse of sGnRH and the medium was changed to normal M199-H-BSA. In short-term tests, the fragments were perifused with medium M199-H-BSA for at least 15 hr at the flow rate of 5 ml/hr before any treatments were given (preincubation) to stabilize the basal release of GTH-II and the flow rate was changed to 15 ml/hr 2 hr before collecting the media in fractions. CGOEs and other tissue extracts were applied for short periods of time (2 or 20 min) as specified in each experiment. In both long-term and short-term tests, the same amounts of BSA were used as control. The perifusion media were collected at 5 min intervals unless otherwise specified and the GTH-II concentrations in the fractions measured with a heterologous carp GTH-II radioimmunoassay as described by Peter *et al.* [25] and Van der Kraak *et al.* [39]

Data analysis

In long-term experiments, the hormone levels in the twelve fractions with the lowest values from each column were averaged and defined as the "basal" level. The hormone levels in all fractions from the same column were expressed as a percentage of this average (% basal) in order to demonstrate any long-term trends in GTH-II secretion, as well as to allow the data from different columns and different experiments to be pooled. In short-term experiments, the data were expressed as either % basal or as a percentage of average levels in the three fractions before each individual treatment was applied (% prepulse). For calculating the hormone responses, the areas under the response peaks were determined. Briefly, the average hormone concentration in the three fractions immediately before each treatment pulse (prepulse, P) was determined. Any value higher than P plus one SEM was considered to be a part of the response peak. P was subtracted from each value in the peak and the differences summed and expressed as a percentage of the basal level of the column, which was defined as the hormone response (GTH-II response).

Statistical analysis was performed by using Student's t-test or one way ANOVA followed by Fisher's least significance difference (LSD) comparison.

RESULTS

Long-term effects of CGOE-mat on GTH-II release

The pituitary fragments in the perifusion column were perifused with CGOE-mat (1 mg/ml) for 24 hr before the perifusates were collected in fractions (pretreatment) and the treatment continued for 2 hr after the collection started (concurrent treatment) (Fig. 1). A series of sGnRH pulses (20 nM, 2 minutes) was applied at 60 min intervals after 24 hr pretreatment with CGOE-mat. CGOE-mat treatment was terminated after the second sGnRH pulse. Pretreatment of goldfish pituitary fragments with CGOE-mat caused an increase in GTH-II release ranging from about 130% to 230% basal. The same amount of BSA in the control columns did not affect the basal levels of GTH-II release. Notably, after terminating perifusion with CGOE-mat and switching to normal medium M199-H-BSA, the basal GTH-II secretion rapidly decreased to the levels (107±26% basal before the third sGnRH pulse) comparable to those in the control columns (110±15% basal before the third sGnRH pulse). In addition to the stimulatory effects on basal GTH-II secretion, continuous perifusion with CGOE-mat also potentiated sGnRH-stimulated GTH-II release; the GTH-II response to the first two sGnRH pulses in the presence of CGOE-mat was significantly greater than in the control columns. The GTH-II response to sGnRH decreased and became comparable to that in the control columns after the switch to normal medium M199-H-BSA.

Short-term effects of CGOE-mat on GTH-II release

To demonstrate whether CGOE has any acute effects on pituitary GTH-II release, CGOE-mat of different doses was applied to goldfish pituitary fragments in perifusion in a pulse of 20 min (Fig. 2). CGOE-mat caused an apparent dose-dependent and rapid release of GTH-II. BSA had no effects on the release of GTH-II from the pituitary fragments.

Effects of other tissue extracts on GTH-II release

Similar to the ovarian preparation, the testicular extract also had strong stimulatory actions on GTH-II release while the ovarian fluid induced a relatively smaller stimulatory

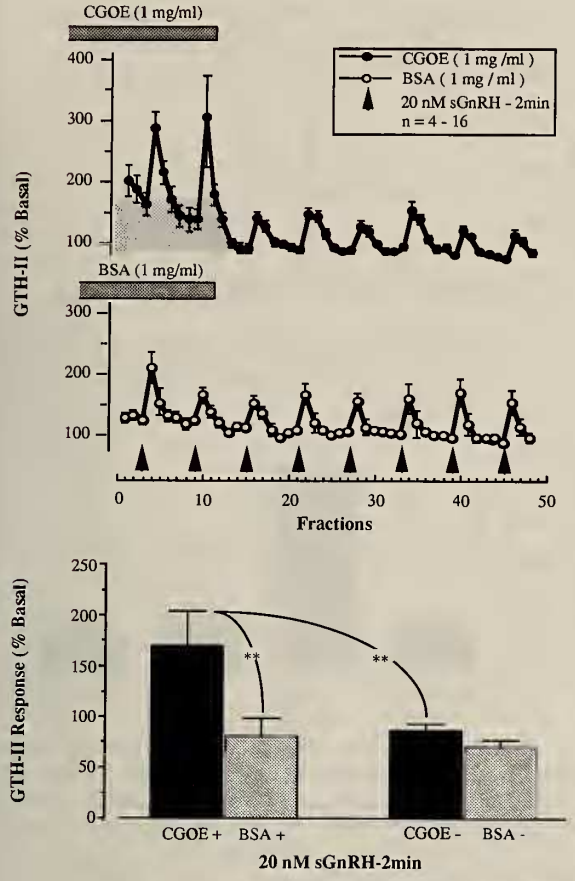

FIG. 1. Long-term effects of CGOE-mat on basal and sGnRH-stimulated goldfish GTH-II release by perifused pituitary fragments from sexually regressed fish. The fragments were perifused with either 1 mg/ml BSA (control) or 1 mg/ml CGOE-mat for 24 hr before collecting the media. A series of 2 min pulses of 20 nM sGnRH was given at 60 min intervals after the fraction collection started. After the second pulse of sGnRH, perifusion with CGOE-mat and BSA was discontinued, and the media changed to normal medium M 199-H-BSA. The perifusate was collected at 10 min intervals. Values are the average of 4–16 columns of separate experiments. The horizontal bars represent concurrent treatments with CGOE-mat and BSA, and the shaded area highlights the elevated basal level of GTH-II. *Lower panel*: GTH-II responses (mean ± SEM, n = 12–56) to sGnRH quantified as described in the Materials and Methods. +, in the presence of CGOE-mat or BSA; −, in the absence of CGOE-mat or BSA. **Significant difference ($p < 0.001$, Student's t-test).

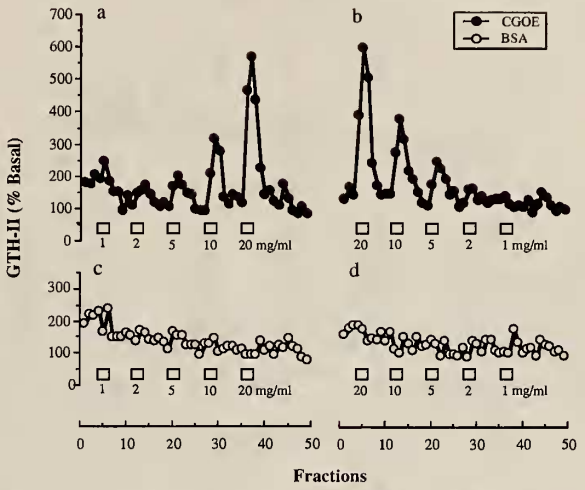

FIG. 2. Short-term effects of CGOE-mat on goldfish. CGOE-mat (a and b) and BSA (control, c and d) were applied for 20 minutes from low dose to high dose (a and c) and from high dose to low dose (b and d) in separate columns, respectively. The perifusate was collected at 10 min intervals. The square symbols represent 20 minute exposure to CGOE-mat or BSA.

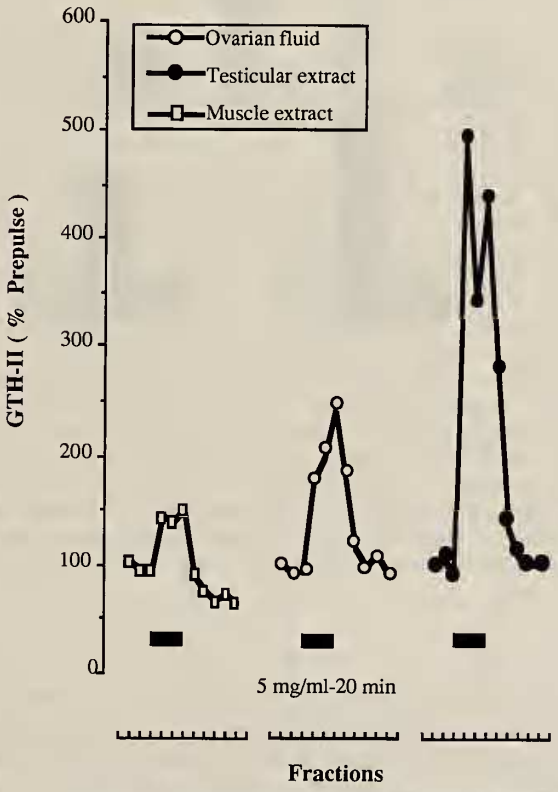

FIG. 3. Effects of extracts from different tissues on goldfish gonadotropin secretion from perifused pituitary fragments. The pituitaries were collected from sexually regressed goldfish. Extracts were applied for 20 minutes at a dosage of 5 mg/ml. The perifusate was collected at 10 min intervals. The values are the average of two columns.

effect. The muscle extract caused only minor increase in GTH-II release (Fig. 3).

Seasonal changes in the effectiveness of the ovarian extracts in stimulating GTH-II release

During the reproductive cycle, the goldfish ovary undergoes major morphological and physiological changes. CGOE from different stages of ovarian development was prepared and tested in the goldfish pituitary fragment perifusion to determine if the *in vitro* stimulatory effects on GTH-II release change with the reproductive cycle. The stimulatory effects of CGOE (5 mg/ml, 2 min) on GTH-II release showed evident seasonality (Fig. 4). Among the three preparations, CGOE-rec was the most effective in stimulating

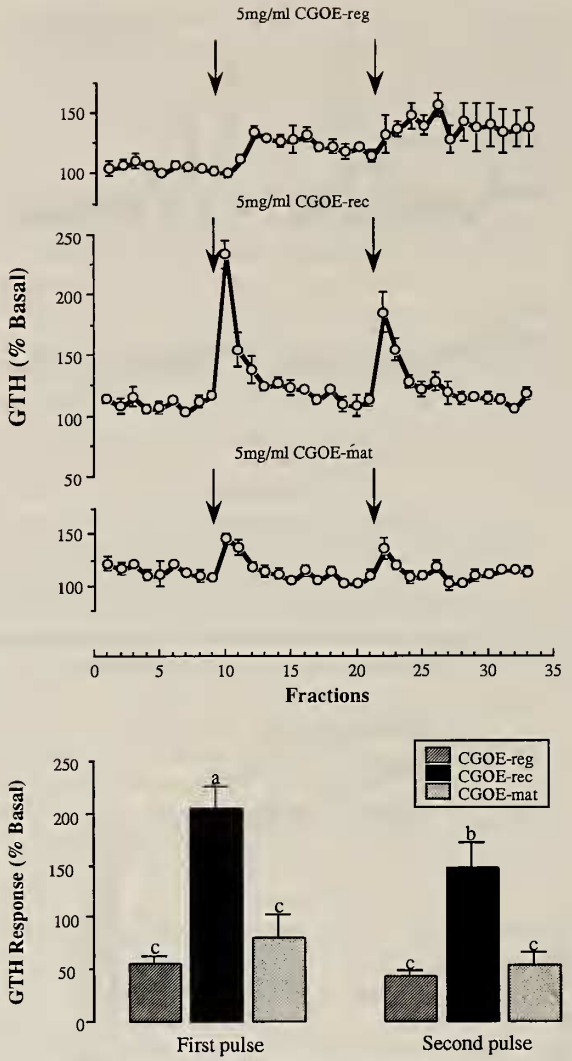

FIG. 4. Effects of CGOE from different stages of ovarian development on goldfish gonadotropin release. The pituitaries were collected from early recrudescing goldfish. The values are the average of four columns. *Lower panel*: GTH-II responses (mean±SEM, n=4) to CGOEs (5 mg/ml, 2 min) from different ovarian stages quantified as described in the Materials and Methods. Different letters above each column indicate significant difference ($p<0.05$, ANOVA followed by LSD comparison).

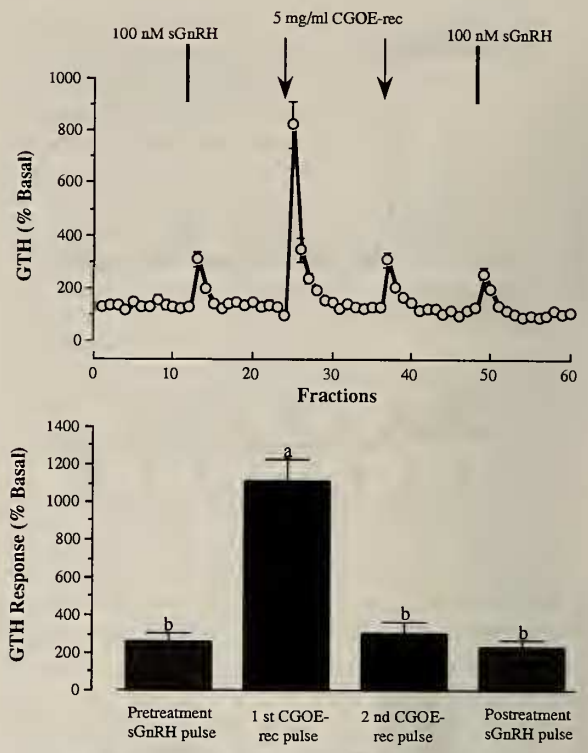

FIG. 5. Effects of CGOE-rec on goldfish GTH-II release by perifused pituitary fragments from recrudescing fish. After overnight preincubation, two 2 min pulses of CGOE-rec were applied to the pituitary fragments at a 60 min interval. sGnRH (2 min pulse) was used as pre- and post-treatment control. Values are the average of five columns. *Lower panel*: GTH-II responses (mean±SEM, n=5) quantified as described in the Materials and Methods. Different letters above each column indicate significant difference ($p<0.05$, ANOVA followed by LSD comparison).

GTH-II release compared to CGOE-reg and CGOE-mat. Interestingly, the pituitary fragments appeared to become refractory to the treatment with CGOE-rec, as the GTH-II response to the second pulse of CGOE-rec significantly decreased compared to that to the first pulse.

The desensitizing effect of CGOE-rec on GTH-II response

The desensitizing effect of CGOE-rec on GTH-II response observed in the above experiment was further confirmed in a separate experiment in which the GTH-II response to CGOE-rec reached a much higher level (Fig. 5). Two sGnRH pulses at the maximal dose (100 nM, 2 min) were given as pre- and post-treatment control. The GTH-II response to the first pulse of CGOE-rec was significantly higher than that to the maximal dose of sGnRH; however, the GTH-II response to the second pulse of CGOE-rec was reduced in magnitude to a level similar to the response to sGnRH. Interestingly, the GTH-II response to the post-treatment sGnRH pulse was not affected by the action of CGOE-rec. The desensitization of the GTH-II response to CGOE-rec was then examined with intervals of 30, 60 and 120 min between the two CGOE-rec pulses (Fig. 6). The desensitizing effect of CGOE-rec continued to occur with a pulse interval of 120 min. The GTH-II responses to the post-treatment sGnRH pulse remained unchanged.

Seasonality of GTH-II responsiveness to CGOE-rec

Two separate experiments were performed using the pituitaries from sexually regressed (gonad weight/total body weight X 100%, gonadosomatic index, GSI=0.90±0.93%; mean±SD) and recrudescing (GSI=3.73±3.59%) goldfish to determine if the seasonal gonadal cycle influences the pituitary GTH-II responsiveness to CGOE-rec (Fig. 7). The GTH-II responses to both sGnRH and CGOE-rec by pituitary fragments from sexually regressed goldfish were

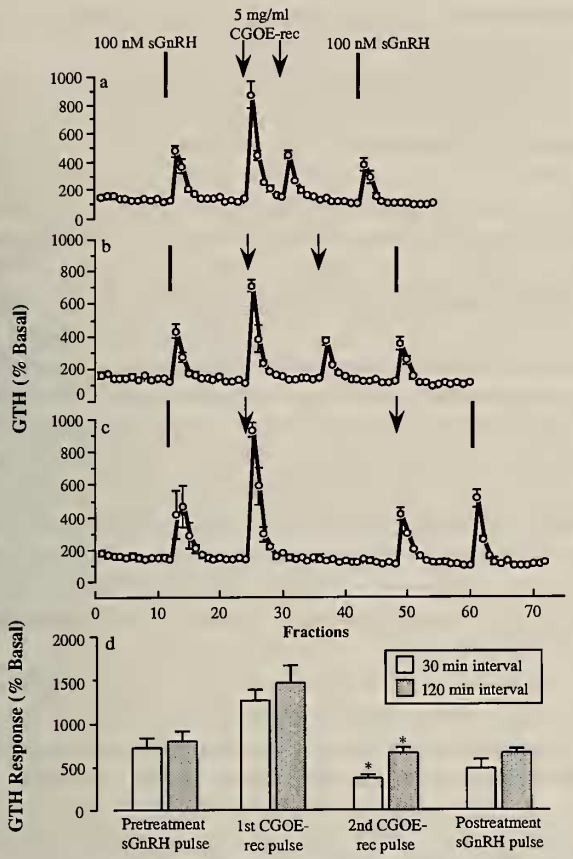

FIG. 6. Effects of CGOE-rec on goldfish GTH-II release by perifused pituitary fragments from recrudescing fish. Two 2 min pulses of CGOE-rec were applied to the pituitary fragments at 30 (a), 60 (b) and 120 (c) min intervals, respectively. sGnRH (2 min pulse) was used as internal pre- and post-treatment control. Values are the average of two (60 min interval) or three columns. *Panel d*: GTH-II responses (mean±SEM, n=3) to sGnRH and CGOE-rec quantified as described in the Materials and Methods. * Significantly different from the corresponding response to the first CGOE-rec pulse ($p<0.05$, Student's t-test). The quantification for the 60 min interval was not shown because only two columns were included in this experiment.

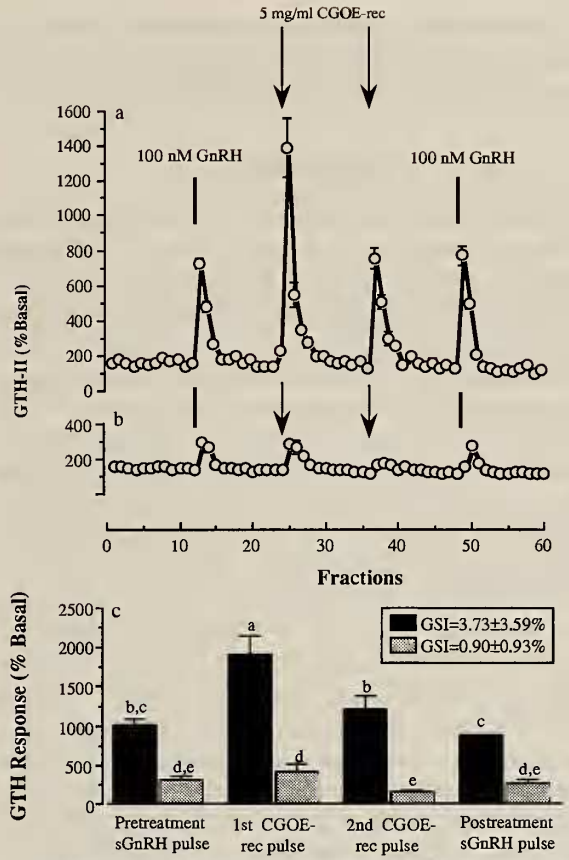

FIG. 7. Effects of gonad developmental stage on the pituitary GTH-II responsiveness to CGOE-rec. Two separate experiments were performed using goldfish at sexually recrudescing (a) and regressed (b) stages. Values are the average of three (a) or four (b) columns. *Panel c*: GTH-II responses (mean±SEM, n=3–4) to sGnRH and CGOE-rec quantified as described in the Materials and Methods. Different letters above each column indicate significant difference ($p<0.05$, ANOVA followed by LSD comparison).

low, and the GTH-II release stimulated by CGOE-rec was similar to that induced by sGnRH. With the pituitaries from sexually recrudescing goldfish, both sGnRH and CGOE-rec caused larger GTH-II responses compared to the pituitaries from regressed fish, with the magnitude of CGOE-rec-induced GTH-II release becoming significantly higher than that of sGnRH-stimulated GTH-II response.

Interaction between dopamine and CGOE-rec on GTH-II release

Dopamine (DA) is a potent hypothalamic inhibitor of basal and sGnRH-stimulated GTH-II release in goldfish [26]. To show whether DA affects CGOE-rec action, the perifused pituitary fragments were exposed to continuous treatment with 1 μM DA during which the first pulse of CGOE-rec was applied (Fig. 8). Treatment with DA caused a slight decrease in basal GTH-II release; however, the GTH-II re-

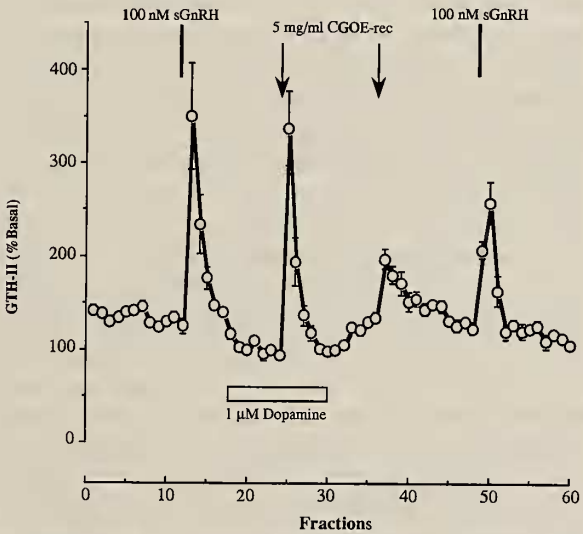

FIG. 8. Effects of dopamine on CGOE-rec-stimulated goldfish GTH-II release. The pituitaries were collected from fully regressed goldfish as those used in Figure 7b. Values are the average of four columns.

sponse stimulated by CGOE-rec was not reduced compared to that in the absence of DA (the GTH-II release by the pituitary fragments from the regressed goldfish in Fig. 7b).

DISCUSSION

Crude aqueous extracts from goldfish ovaries exhibit stimulatory effects on both basal as well as sGnRH-stimulated GTH-II release after long term treatment. Furthermore, the extracts from both goldfish ovaries and testes have acute stimulatory actions on pituitary GTH-II release in perifusion, with pulsatile treatment for as short as 2 minutes eliciting a rapid response of GTH-II release. This is in contrast to mammalian studies on gonadal fluids and extracts that generally have inhibitory effects on pituitary GTH, expecially FSH, release, attributed to the actions of inhibin [41]. Another feature of the present results that is distinct from those in mammalian species is the acute response to CGOE and testicular extract. Although the acute effects of CGOE appear to be similar to that induced by GnRH, some evidence indicates that the novel stimulatory factor(s) in CGOE are different from GnRH. First, the extracts had been subjected to dialysis with 12 kD cut-off point to remove small molecules including small peptides; therefore, the putative stimulatory factors in the foldfish gonadal extracts are likely to have molecular weight of more than 12 kD. Second, even though CGOE-rec exhibits a strong desensitizing effect on the GTH-II response to the subsequent treatment, the desensitization of the response to CGOE-rec does not reduce the GTH-II response to sGnRH stimulation, suggesting that the desensitization is specifically related to the actions of CGOE-rec and is independent of sGnRH.

One interesting aspect of the GTH-II-releasing activity of goldfish ovarian extracts is that the stimulatory actions change significantly with the developmental stages of the ovary, with CGOE-rec being the most effective compared to CGOE-reg and CGOE-mat. Furthermore, the pituitary responsiveness to CGOE-rec also varies significantly with the development of gonads. The GTH-II responsiveness to CGOE-rec increases significantly when the fish enter sexual recrudescence and maturation, similar to the seasonal variations in responsiveness to GnRH in goldfish (C.K. Murthy and R.E. Peter, unpublished results); however, the increase in the GTH-II response induced by CGOE-rec is even higher. The increase in the action of CGOE-rec compared to CGOE-reg and CGOE-mat, and the significantly higher GTH-II responsiveness to CGOE-rec by the pituitaries from sexually recrudescing fish correlate well with the onset of ovarian recrudescence and the associated increase in plasma GTH-II levels [11, 24, 37], implying that the stimulatory factor(s) in CGOE-rec might be involved in initiating these changes.

Interestingly, although DA is potent in suppressing basal and GnRH-induced GTH-II secretion in goldfish [26], DA does not affect CGOE-rec-stimulated GTH-II release. This suggests that there may be a stimulatory pathway for GTH-II in goldfish that is independent of the tonic inhibition by the hypothalamic DA.

In mammals, a variety of factors have been identified in gonadal preparations that can either stimulate or inhibit pituitary GTH release [14, 18, 27, 31, 32, 34, 41, 42]. Therefore, any observed actions of a crude preparation only reflect a net effect depending on the relative amounts, potencies and the complex interactions of all active substances present in the mixture. This also applies to the goldfish gonadal extracts used in the present study. With overwhelming evidence in mind for the inhibitory effects of mammalian gonadal preparations on pituitary GTH secretion, the stimulatory actions of the goldfish gonadal extracts on pituitary GTH-II release described above are unexpected, although it has been reported that *Tilapia* seminal plasma and testicular extract are also stimulatory to GTH release from the pituitary fragments in perifusion [30]. The nature of the putative stimulatory factors in goldfish gonads remains unknown and additional studies are needed to identify these factors and characterize their biochemical and biological properties.

Our previous studies showed that inhibin and activin-containing porcine follicular fluid (pFF), purified porcine inhibin A and activin A have stimulatory actions on goldfish GTH-II release [8]. The stimulatory effects of porcine inhibin on goldfish GTH-II release is in sharp contrast to its well-documented inhibitory actions in mammalian species. Furthemore, both porcine inhibin and activin have acute effects on goldfish GTH-II release in pituitary fragment perifusion, and their actions are not suppressed by DA. Interestingly, inhibin and activin-containing pFF is surprisingly similar to CGOE in terms of long-term and short-term effects on GTH-II release in goldfish, and both pFF and CGOE-rec have strong desensitizing effects on GTH-II response [8]. Using domain-specific antibodies, the immunoreactive inhibin and activin subunits have been demonstrated in the goldfish gonads [9]. We have recently cloned and sequenced goldfish activin βA and βB subunits, and demonstrated that inhibin and activin β subunits are highly conserved throughout vertebrates [10]. Toghther with the results presented in the present paper, these previous results suggest that inhibin and activin-like molecules in the goldfish gonads may, at least partially, contribute to the unique stimulatory actions of goldfish gonadal extracts.

ACKNOWLEDGMENTS

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